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PRINCIPAL INVESTIGATOR: Charles N. Fehr
Robert Gillies, Ph.D.

CONTRACTING ORGANIZATION: The University of Arizona
Tucson, Arizona 85719

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6. AUTHOR(S)
Charles Fehr
Robert Gillies, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Arizona
Tucson, Arizona 85719

E-Mail: fehr@u.arizona.edu

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Vacuolar-type ATPases are heteromultimeric proteins found in all eukaryotic cell types. Thel15kD subunit is a membrane-bound componentof unknownfunction. The subunit has been cloned, and PCR products coding for non-transmembrane domains generated for ligation into a fusion protein expression vector and subsequent fusion protein and antibody production. Agarose gel electrophoresis of post-ligation DNA suggests inserts have been ligated into the expression vector, however no fusion protein expression has beensuccessfully induced. Northern analysis of cancer cell lines xpressing V-ATPase activitygrown at different pH'ssuggests that 115kD subunit mRNA levels are not altered by incubation of cells at acidic pH.

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FOREWORD

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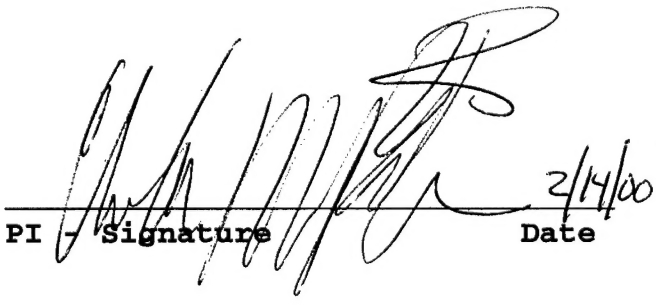

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Introduction

Vacuolar type H^+ ATPases (V-ATPase) are heteromultimeric proteins found in all eukaryotic cells, and have been highly conserved among divergent species. V-ATPases couple hydrolysis of ATP to the translocation of protons across a membrane and are largely responsible for the acidification of intracellular vesicles and membrane-bound compartments. In addition, some cell types express V-ATPases at the plasma membrane, where they may play a role in pH regulation or the acidification of extracellular compartments.

The pump complex is comprised of "V_1" and "V_0" regions (by analogy with the related F_1F_0 H^+ ATPases). V0 is a hydrophobic, integral membrane region comprised of a hexamer of 16kD subunits and single copies of 115, 45, and 39kD peptides. The 16kD hexamer is thought to form the proton "pore'. V1 contains a hydrophillic "head", composed of three copies each of A (70kD) and B (60kD) subunits, and an intervening "stalk", with single copies of C (41kD), D (34kD), and E (33kD) subunits. Both the A and B subunits have ATP binding sites, with catalysis thought to occur on the A subunit, and the ATP binding properties of the B subunit proposed to be regulatory. The specific function of other subunits is unknown. Although ATP catalysis of both C and E subunits, these and other subunits are thought to mediate V1-V0 association and/or play other regulatory roles.

The existence of different isoforms of various pump subunits has been demonstrated, and it has been proposed that these alternative isoforms may result in cell- or organelle-specific targeting or specificity of activity of VATPases in different tissue types or subcellular compartments. For example, osteoclasts, which constituitively express VATPase at the plasma membrane (for acidification of extracellular bone resorptive compartments), have been shown to express different

isoforms of A and B (Chatterjee et al 1992) and 115 kD (Li et al 1996) subunits. These isoforms may provide for specific targeting and regulation of plasma membrane VATPase activity distinct from that of "housekeeping" VATPase activity in endocytic compartments, Golgi, lysozomes, etc. Alternative splicing has been shown to give rise to different isoforms of the A and 115kD (Peng et al 1994) subunits in a tissue-specific manner, and distinct genes encode different isoforms of the B and 115kDsubunits. Thus far, cell types expressing unique subunit isoforms are those in which altered VATPase activity has been demonstrated or hypothesized i.e. extracellular compartment acidification by osteoclasts or renal epithelia, synaptic vesicle acidification in brain. In contrast, the classical, ubiquitous isoforms are present in most cell types. Demonstration that distinct genes code for alternative forms of the 115kD homolog in yeast (Manolson et al 1994) suggests that this subunit may play a role in targeting or regulation of the pump within a single cell. Other subunits with unknown function- C, D, E, 39kD, 45kD, etc., may also be involved in regulation, but well-developed models of VATPase regulation are currently lacking.

A number of cell types express VATPases at the plasma membrane. In these cells, including osteoclasts, macrophages, renal epithelia and neural cells, the plasmalemmal VATPase expression is important to some specialized cell function. Osteoclasts use the pump to acidify an extracellular bone resorptive compartment during skeletal development. Macrophages, which may recruit up to 50% of plasma membrane during phagocytosis, constituitively express VATPase at the plasma membrane, likely in order to facillitate rapid phagosomal acidification. Apical plasma membrane of renal intercalating cells is highly enriched in VATPase for H+ export into the urine, and in neurons rapid acidification of plasma membrane-derived synaptic vesicles is neccesary for effective concentration of neurotransmitter.

In addition to the aforementioned cell types, a large number of cancerous cell lines exhibit plasma membrane VATPase activity, as demonstrated by a bafilomycin-sensitive ability to recover from an acid load in absence of Na^+ and HCO_3^- (Martinez-Zaguillen et al 1992). The significance of this activity in cancerous cells is not clear, but a number of possibilities exist. VATPases are not usually thought to be significantly involved in cytosolic pH maintenence, as VATPase activity is low with respect to the Na^+-H^+ exchanger (NHE). However, VATPases clearly have the potential to significantly affect cytosolic pH, especially at alkaline values , where NHE activity is diminished. Proliferative and cancerous cells generally maintain a higher cytosolic pH than do normal cells, even within tumors where localized extracellular pH may be very acidic. Plasmalemmal VATPase activity could be significant in allowing cells to maintain this exagerrated pH gradient, sustaining cytosolic conditions conducive to growth, and even selecting for these cells in acidic environs.

Our attempts to visualize VATPase at the plasma membrane in cells expressing this activity via immunocytochemistry have been inconsistent at best. This suggests the possibility that this activity may be dynamic, i.e. involving rapid cycling of VATPase-containing vesicles with the plasma membrane. Such a mechanism would allow for exocytotic removal of protons from the cell, while not requiring extensive, static residence of the VATPase in the plasma membrane itself.

As was described in last year's report and the revised Statement of Work, our aim is to generate fusion proteins using PCR-amplified sequences of the 115kD subunit. These fusion proteins will be used to determine the specificity of OSW-2, a monoclonal antibody targeting the 115kD subunit. These fusion proteins will also be used to generate antibodies against domains of the 115kD subunit., These antibodies will be used to a) epitope map the 115kD subunit relative to its orientation in the membrane, b) determine by Western analysis if the 115kD protein is upreguated in response to acidic environment in a number of breast cancer cell lines, c) determine

by antibody uptake experiments the effect of blocking endocytosis on plasmalemmal VATPase activity. As our model predicts that the VATPase activity seen in cancer cell lines expressing this activity is due to rapid cycling of VATPase-containing vesicles, we predict that interfence with endocytosis will block plasmalemmal VATPase activity in these cells, and may reverse the drug resistance that correlates with functional plasma membrane VATPase activity in a number of breast cancer cell lines.

Body

Though not for lack of effort, progress towards goals set forth in the Statement of Work was minimal at best during the final year of this grant. Much of the proposed work was dependent upon production of fusion proteins mimicking various domains of the 115kD subunit. I was never able to accomplish this. Despite having PCR products coding for the desired protein stretches, I was never able to successfully ligate and express the fusion proteins within the expression system we employed. The PCR primers were designed to have overhanging sequences allowing for restriction digestion and subsequent ligation into the expression vector p-GEX 2T. Despite innumerable attempts the only expression induced was that of glutathione S-transferase, the built-in vector protein. In the course of attempting to accomplish proper expression the PCR primer sequences were analyzed independently by a colleague and myself, and both agreed that the sequences were correct. New primers were ordered but the same lack of proper expression was continually encountered Due to the dependence of most of the proposed work on successful protein expression and subsequent antibody production, attempts to accomplish this technically trivial (supposedly) step were made *ad nauseum*. The fallout of my failure to succeed in this particular set of experiments is addressed in an appended letter. One stated goal was not dependant upon fusion protein production, that of examining 115kD mRNA levels in cells grown at different pH . MCF-10T and the parental MCF-10A line were each grown at pH 6.8 and 7.4 and Northern analysis done using a 1.1 kb PCR product coding for the N-terminus of the 115kD subunit. No significant differences were detected in these experiments, consistent with previous

findings suggesting that overall expression of the V-ATPase is not altered by growth in acidic conditions. Though these results were not what I'd hoped, the successful use of the probe strongly suggested that the problem with the fusion protein expression was one directly related to proper ligation with the pGEX vector. As a result of these findings and the lack thereof, no truly significant results or publications arose from this work. It is my hope and understanding that the project will be picked up and continued by another worker in my former lab.

Appendix I

I include this letter as a personal note. Despite my lack of success in this work, including my decision (in the face of physical and psychological trauma related to my frustration) to forego a research career in favor of teaching, I want to state emphatically that the Army Breast Cancer Initiative did not err in awarding me this grant. At the time of the award I was highly motivated and had what I still believe to be a very interesting and thoughtful project. My naivete concerning the patience necessary to conduct scientific research, combined with the technical problems I encountered was something that I did not foresee, and those awarding me this grant certainly couldn't have. My desire to do this work on my own and my reluctance to seek out aid and advice from other labs is something that contributed to my failure and is something I regret. The fight against breast cancer is one I take personally, as a number of women in my family including my mother have fought this disease. I know that the experience I gained while working on this project will aid me as I endeavor to teach young men and women to respect and care for their bodies in a high school setting. It is my expectation that your initiative, though designed to foster basic and applied research, has in my case resulted in a teacher able to relay the importance of taking preventative steps to my students. It is my hope that this dynamic is not lost on those of you working on this initiative. Thank you very sincerely for your support.